Identification of complement factor 5 as a susceptibility locus for experimental allergic asthma

Christopher L. Karp[1,*], Andrew Grupe[2,*], Eric Schadt[2], Susan L. Ewart[3], Michelle Keane-Moore[4], Peter J. Cuomo[1], Jörg Köhl[5], Larry Wahl[6], Douglas Kuperman[4], Soren Germer[7], Dee Aud[2], Gary Peltz[2] and Marsha Wills-Karp[4]

The prevalence and severity of allergic asthma continue to rise, lending urgency to the search for environmental triggers and genetic substrates. Using microarray analysis of pulmonary gene expression and single nucleotide polymorphism–based genotyping, combined with quantitative trait locus analysis, we identified the gene encoding complement factor 5 (*C5*) as a susceptibility locus for allergen-induced airway hyperresponsiveness in a murine model of asthma. A deletion in the coding sequence of *C5* leads to C5-deficiency and susceptibility. Interleukin 12 (IL-12) is able to prevent or reverse experimental allergic asthma. Blockade of the C5a receptor rendered human monocytes unable to produce IL-12, mimicking blunted IL-12 production by macrophages from C5-deficient mice and providing a mechanism for the regulation of susceptibility to asthma by *C5*. The role of complement in modulating susceptibility to asthma highlights the importance of immunoregulatory events at the interface of innate and adaptive immunity in disease pathogenesis.

The worldwide prevalence and severity of allergic asthma have increased dramatically in recent decades. Unfortunately therapeutic advances have not kept pace, and asthma morbidity and mortality continue to rise[1]. The cardinal features of allergic asthma include airway hyperresponsiveness (AHR) to a variety of specific and nonspecific stimuli, excessive airway mucus production, pulmonary eosinophilia and elevated concentrations of serum immunoglobulin E (IgE). It is generally accepted that asthma arises as a result of inappropriate immunological responses to common environmental antigens in genetically susceptible individuals[2]. Pathophysiology is thought to be mediated by $CD4^+$ T lymphocytes producing a type 2 cytokine profile[2]. The molecular mechanisms that underlie susceptibility to these aberrant immune responses are unknown. A genetic approach has promised insight into mechanism. Family studies demonstrate a heritable predisposition to asthma. The mapping of asthma susceptibility genes in humans has been hampered by variability in phenotype, genetic heterogeneity across populations and uncontrolled environmental influences. Despite this, more than 20 linkage regions have been found[3–6], although the path from linkage to gene remains untraveled to date.

A well characterized murine model that mimics the pathophysiology of human allergic asthma was used in a reductive approach to these complexities[7]. In this model, allergen exposure results in AHR, increased airway mucus content, antigen-specific IgE in serum, and pulmonary eosinophilia[2,7]. As with human asthma, the pulmonary inflammatory response in experimental asthma centers around the airway wall and involves extensive airway wall remodeling, including: goblet cell metaplasia; thickening of the airway epithelial layer; and airway smooth muscle hypertrophy. Inbred mouse strains vary in their susceptibility to disease induction in this model. Allergic airway

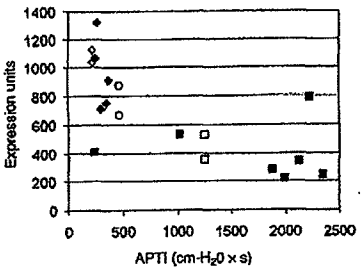
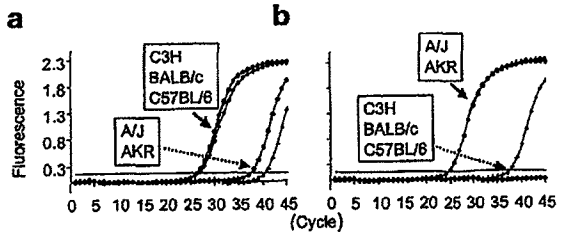


Figure 1. *C5* gene expression and genotype correlate with allergen-induced AHR. C5 transcript levels in whole lungs of OVA-sensitized A/J (□), C3H/HeJ (◇) (A/J × C3H/HeJ)F_1 (○), 6 BC_1-high responder and 6 BC_1-low responder mice are shown in comparison with their dynamic airway pressure (APTI in cm-H_2O × s). BC_1 mice carrying only the A/J *C5* allele (■); BC_1 mice that are *C5* heterozygous (♦). The outlier BC_1 mouse (homozygous for the A/J *C5* allele, low APTI) is discussed in the text. Expression units reflect the average difference value as analyzed by GeneChip 3.01 Software.

[1]*Department of Medicine, Johns Hopkins University, School of Medicine, Baltimore, MA, USA.* [2]*Roche BioScience, 3401 Hillview Avenue, Palo Alto, CA 94304–1397, USA.* [3]*Department of Large Animal Sciences, Michigan State University, B41 National Food Safety and Toxicology, East Lansing, MI 48824–1302, USA.* [4]*Department of Environmental Health Sciences, Johns Hopkins University School of Hygiene and Public Health, Baltimore, 615 N. Wolfe St., Baltimore, MD 21205, USA.* [5]*Institut für Medical Microbiology, Medizinische Hochschule Hannover, Carl-Neubergstr. 1, 30625 Hannover, Germany.* [6]*National Institute of Dental and Craniofacial Research, 30 Convent Drive, Bethesda, MD 20892 USA.* [7]*Roche Molecular Systems, Inc., 1145 Atlantic Atlantic Avenue, Alameda CA 94501, USA.* **These authors contributed equally to this work.*
Correspondence should be addressed to M.W.-K. (mkarp@welch.jhu.edu).



	C5	APTI (cm-H_2O × s)	N
A/J	-/-	1207 (70)	10
AKR/J	-/-	1185 (328)	6
C3H/HeJ	+/+	282 (24)	14
BALB/cJ	+/+	628 (40)	6
C57BL/6J	+/+	340 (48)	6

Figure 2. *C5* allele correlates with allergen-induced AHR across inbred strains. *A/J* have a 5′ deletion in the *C5* gene and carry the *C5-D* allele; C3H/HeJ mice do not have the deletion and carry the *C5-S* allele. Allele-specific kinetic polyacrylamide gel electrophoresis (PCR) was used to genotype other inbred mouse strains. (a) Kinetic PCR using the C3H/HeJ allele-specific primer identifies the *C5-S* allele in three strains. Primer dimer formation is detected in strains lacking this allele after amplification cycle 36. (b) Kinetic PCR using the A/J allele-specific primer identifies the *C5-D* in two strains. (c) Dynamic airway pressure (APTI in cm-H_2O × s) following allergen-sensitization and challenge for these five inbred murine strains. APTI measurements represent means ± s.d. in parentheses of the indicated number of mice for each strain.

responses are mediated by cytokines produced by type 2 $CD4^+$ T cells in susceptible strains of mice. Congruent with the well defined role of interleukin 12 (IL-12) in promoting type 1 $CD4^+$ T cell differentiation, disease is prevented or ablated by exogenous IL-12 in susceptible strains[8]. Similarly, neutralization of IL-12 renders resistant strains susceptible to disease[9]. Previous studies have shown that the various features of the asthmatic phenotype are separable, both genetically and experimentally in this model. Two strains with markedly different susceptibilities to experimental allergen-induced AHR, the primary phenotypic signature of human asthma, were used in a search for genetic substrates.

Results

C5 as a candidate gene

To identify gene candidates, pulmonary gene expression was profiled with oligonucleotide microarrays. A/J (highly susceptible to allergen-induced AHR) and C3H/HeJ (highly resistant) mice were immunized intraperitoneally and were subsequently challenged intratracheally with soluble ovalbumin (OVA); the allergic phenotype was assessed 3 days after the antigen challenge. After phenotypic assessment, lungs were collected from A/J, C3H/HeJ (A/J × C3H/HeJ)F_1 (called hereafter F_1) and eight of the segregating F_1 × A/J backcross (BC_1) mice that exhibited phenotypically extreme allergen-induced airway responsiveness (APTI). (APTI of BC_1-high, 1280–2349 cm-H_2O × s; APTI of BC_1-low, 256–514 cm-H_2O × s.) Of the 7350 genes on the microarray, 2718 were expressed in the lungs of the parental strains and 739 were differentially expressed in the lungs of A/J and C3H/HeJ mice. Approximately 227 genes exhibited a greater than threefold change in expression when these two parental strains were compared.

Differential gene expression was assessed within groups of high- and low-responder mice by comparing expression in four BC_1-high responder and A/J mice, and four BC_1-low responder and C3H/HeJ mice, respectively. The expression data was filtered for differential expression in four of the five intragroup pairwise comparisons, as suggested by the Affymetrix GeneChip software. This analysis yielded 21 differentially expressed genes. Previous analysis of the inheritance pattern of allergen-induced AHR in these strains led to the identification of two distinct quantitative trait loci (QTL) on chromosome 2: *Abhr1* ("allergen-induced bronchial hyperresponsiveness") and *Abhr2* (lod scores=4.3 and 3.7, respectively)[10]. *C5*, located near *Abhr2* at 23.5 cM on chromosome 2, was the only gene that both met the differential expression criteria and was located in one of the defined QTL intervals. No gene within the *Abhr1* support interval satisfied the differential expression criteria.

The initial expression data set was confirmed by expression profiling of additional BC_1-high responder (n=2), BC_1-low responder (n=2) and parental (A/J and C3H/HeJ) mice, to yield a total of 18 data points. Direct comparison of allergen-induced AHR with pulmonary C5 mRNA expression in A/J, C3H/HeJ, F_1 and 12 BC_1 mice exhibiting extreme allergen-induced AHR phenotypes revealed that: resistant (C3H/HeJ and BC_1-low responder) mice had high *C5* gene expression; susceptible (A/J and BC_1-high responder) mice had low *C5* gene expression; and F_1 mice had intermediate *C5* gene expression (Fig. 1). The amount of C5 expression was significantly associated with genotype (P<0.005 for the BC_1 mice alone; P<0.001 including the parental and F_1 mice as well, with Student's t-test).

C5 expression also correlated with the magnitude of allergen-induced AHR, linear correlation coefficient (r^2) = -0.66. The *C5* genotype was

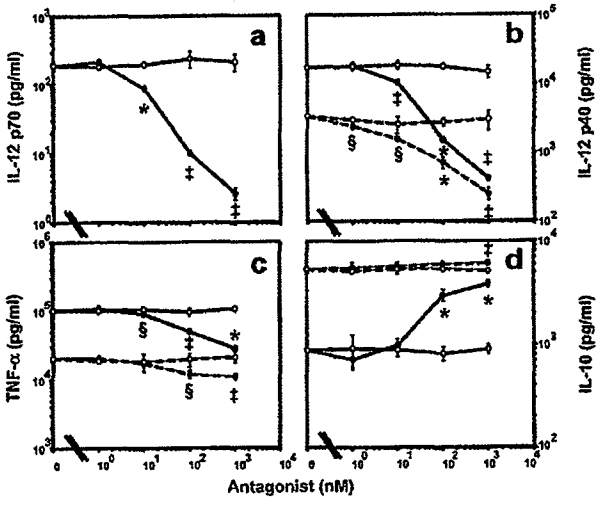


Figure 3. Effects of C5aR blockade on cytokine production by primary human monocytes. Monocytes were incubated in serum-free conditions with the indicated concentration of C5aR antagonist (•) or control (○). Stimulation was with IFN-γ + SAC (solid line) or SAC alone (dashed line). (a) IL-12 p70 production. (b). IL-12 p40 production. (c) TNF-α production. (d) IL-10 production. Values shown are means ± s.d. in a representative experiment (n=4). Statistical analysis was done with paired t-tests. *P<0.0005, ‡P<0.005, §P<0.05. At 1 μM of the C5aR antagonist, there was 95.4% (s.e. 3%) mean inhibition of IL-12 p70 production; 91.7% (s.e. 6.3%) inhibition of IL-12 p40 production to stimulation with IFN-γ + SAC, 91.6% (s.e. 4.6%) to SAC alone; 63.9% (s.e. 5.7%) inhibition of TNF-α production to IFN-γ + SAC, 51.3% (s.e. 6.1%) to SAC alone; and 254% (s.e. 64.8%) mean augmentation in IL-10 production after stimulation with IFN-γ + SAC, -0.4% (s.e. 14.7%) after SAC alone (n=4).

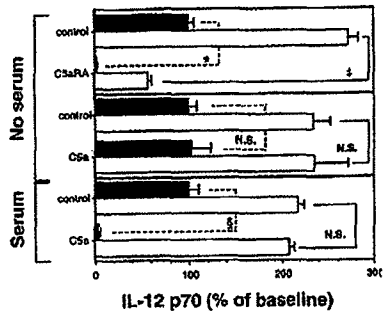


Figure 4. C5aR signaling and C5aR blockade have mechanistically distinct effects on IL-12 production: serum dependence and pertussis toxin sensitivity of signaling. Monocytes were incubated in media without (filled bars) or with (open bars) 250 ng/ml of pertussis toxin in the presence or absence of 10% fetal bovine serum for 2 h. Cells were subsequently cultured (with or without serum) in the presence of 100 nM C5aR antagonist or 100 nM recombinant C5a (Sigma), or appropriate controls (control lysate[18] for C5aRA, media for C5a). Stimulation was with IFN-γ + SAC. Data presented are means ± s.d. in a representative experiment (n=3). Statistical analysis was done by paired t-test. *P=0.000007, †P=0.000004, ‡P=0.00006.

analyzed for all BC_1 mice shown in **Fig. 1**. Five out of six BC_1-low responder mice were heterozygous at the *C5* locus, and had high pulmonary C5 mRNA expression. The single BC_1-low responder mouse with low pulmonary C5 mRNA expression was homozygous for the A/J allele at the *C5* locus, a result that is congruent with the QTL analysis demonstrating the presence of at least two loci controlling allergen-induced AHR in this model.

SNP-based genotypic analysis

The relationship between *C5* expression and genotype has been examined previously in murine systems. A/J mice have a 2-bp deletion in a 5′ exon of the *C5* gene that renders them deficient in C5 mRNA and protein production (and devoid of functional C5); C3H/HeJ mice are C5-sufficient[11]. The link between C5 and AHR was explored further by characterizing the correlation between *C5* genotype and susceptibility to allergen-induced AHR. A high-throughput single nucleotide polymorphism (SNP)-based genotyping assay, employing *C5* allele-specific oligonucleotide primers and kinetic thermal cycling, was used to genotype 172 BC_1 mice with phenotypically extreme allergen-induced AHR responses.

BC_1-high responder mice tended to be homozygous for A/J *C5* alleles (*C5-D*); whereas BC_1-low responder mice tended to be heterozygous, carrying both *C5-D* and C3H/HeJ (*C5-S*) alleles (**Table 1**). These differences in *C5* allele frequency were statistically significant, confirming the correlation between *C5* genotype and susceptibility to allergen-induced AHR in the progeny of these parental strains. The correlation between phenotype and genotype at this locus is not absolute, confirming the presence of multigenic influences on allergen-induced AHR. As a control, these same BC_1 mice underwent SNP-based genotyping of the Toll-like receptor 4 gene (*Tlr4*) on chromosome 4, a dominant negative mutation in which is responsible for endotoxin hyporesponsiveness in C3H/HeJ mice[12,13]. No significant difference in *Tlr4* allele frequency was found between the high- and low-responder populations (**Table 1**).

To begin to assess the breadth of the linkage between *C5* genotype and susceptibility to allergen-induced AHR, SNP-based genotypic analysis was done in other, previously phenotyped, inbred murine strains (**Fig. 2**). The correlation held fast in this limited sample of strains. Both of the susceptible strains (AKR/J and A/J) carry the deletion in *C5*, whereas resistant (C57/BL/6J, BALB/cJ, C3H/HeJ) strains do not carry this deletion.

C5a effects on IL-12 production

C5 cleavage fragments exert pleiotropic effects on inflammatory responses. C5a has been shown to stimulate the production of proinflammatory cytokines by monocytes and macrophages. The effects of C5a on monocytic production of IL-12, a T helper cell subset 1 (T_H1)-promoting proinflammatory cytokine that is critical to regulation of the asthmatic phenotype in this model, were therefore examined. *In vitro* analysis of the role of complement in modulation of monocyte and macrophage function is complicated, experimentally, by the facts that: such cells synthesize the complement system autologously; complement activation occurs readily *in vitro*; and the use of serum provides access to complement activation fragments including C5a[14–17]. Therefore, to mimic the *in vivo* lack of access of A/J macrophages to C5a, we studied the effects of ablating C5a-mediated signaling in human monocytes cultured in the absence of exogenous complement. A potent, specific C5a receptor (C5aR) antagonist, isolated by panning C5a COOH-terminal libraries on a C5aR-expressing cell line, was used[18]. This antagonist binds to human leukocytes with an median inhibitory dose (ID_{50}) in the low nanomolar range and exhibits no detectable agonist activity even at micromolar concentrations[18].

Human monocytes were incubated under serum-free conditions with immunoaffinity-purified C5aR antagonist (or a control lysate similarly purified from bacteria not expressing recombinant C5aR antagonist), and secondarily stimulated for monokine production. Blockade of the C5aR caused marked, dose-dependent inhibition of the IL-12 productive capacity of primary human monocytes (**Fig. 3a,b**). This occurred both at the level of the functional p70 heterodimer and at the level of the highly regulated p40 subunit. K-76, a complement-inhibitory monocarboxylic acid derived from fungi with primary effects on C5 activation[19], also inhibited IL-12 production by monocytes (data not shown). We examined the specificity of these effects by characterizing the effects of C5aR blockade on the principal pro- and anti-inflammatory cytokines produced by monocytes and macrophages: tumor necrosis factor α (TNF-α) and IL-10. C5aR blockade also inhibited the secretion of TNF-α, albeit with less potency than its effects on IL-12 (**Fig. 3c**). Although inhibition of C5a-mediated signaling had no overall effect on bacterially driven IL-10 production, IFN-γ–mediated suppression of IL-10 production was reversed (**Fig. 3d**). Taken together, these latter findings suggest that functional C5-deficiency leads to a relatively anti-inflammatory phenotype, especially in the presence of the type 1 cytokine IFN-γ.

Table 1. *C5* genotype correlates with allergen-induced AHR in BC_1-high responder and BC_1-low responder mice

alleles	Number of mice with allele type	
	BC_1-high	BC_1-low
C5 genotype		
AA[a]	54	32
AC[b]	32	54
Tlr4 genotype		
AA	36	46
AC	50	40

[a]AA, homozygous A/J allele. [b]AC, heterozygous (A/J, C3H/HeJ) alleles. Template DNA was prepared from 172 BC_1 mice exhibiting extreme phenotypes for allergen-induced AHR (APTI value for BC_1-high, 1280–2349 cm-H_20 × s; APTI for BC_1-low, 256–514 cm-H_20 × s). Genotyping was done by kinetic PCR with specific primers for A/J and C3H/HeJ alleles of *C5* and *Tlr4*. Statistical analysis was done using the Fisher exact test. *C5* genotype: P=0.0013. *Tlr4* genotype: P=0.169.

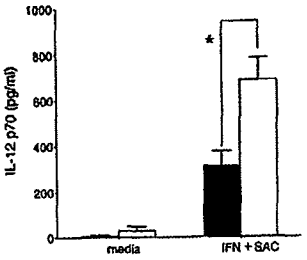


Figure 5. IL-12 production by macrophages from A/J and C3H/HeJ mice. Peritoneal macrophages from A/J (filled bars) and C3H/HeJ (open bars), isolated after thioglycollate elicitation, were stimulated with IFN-γ + SAC, or mock-stimulated with media. After 24 h, IL-12 p70 production was measured by enzyme-linked immunosorbent assay (ELISA) (Pharmingen) in cell-free supernatants. Data presented are means ± s.e. of 11 mice per strain. Statistical analysis was done by paired *t*-test. *$P<0.0001$.

It has recently been reported that C5a can itself lead to downmodulation of IL-12 production under certain conditions[20,21]. These data are replicable (**Fig. 4**). However the inhibition of IL-12 production induced by C5a is clearly and mechanistically separable from that resulting from ablation of C5aR signaling. The C5aR is coupled to a pertussis toxin–sensitive G protein[22]. Notably, C5a-mediated inhibition of IL-12 production is pertussis toxin sensitive and only occurs in the presence of serum. In contrast, C5aR antagonist-mediated inhibition is not pertussis toxin–sensitive and does not depend upon exogenous serum (**Fig. 4**).

To further investigate the relevance of these observations to experimental allergic asthma, IL-12 production was compared in A/J and C3H/HeJ mice. Peritoneal macrophages from C5-deficient A/J mice produce significantly less IL-12 than those obtained from C5-sufficient C3H/HeJ mice (**Fig. 5**).

Discussion

We previously identified two QTL for allergen-induced AHR on chromosome 2, *Abhr1* and *Abhr2*[10]. To make the transition from QTL to gene, we took advantage of recent technological advances that have made genetic analysis of complex traits increasingly tractable in experimental models. QTL analysis was combined with microarray analysis of target organ gene expression and SNP-based genotyping to identify *C5* as a susceptibility locus for AHR. Genotypic analysis of the phenotypically diverse backcross (BC_1) mice was critical to investigation of the link discovered between phenotype, gene expression and genotype. The power of gene array analysis is tempered by its inability to distinguish between primary and secondary effects on gene expression. SNP analysis of the backcross animals demonstrated the presence of a genetic polymorphism at the *C5* locus with a primary effect on the phenotype of allergen-induced AHR. The association identified between the *C5* locus and AHR in our study was not complete, accurately reflecting the multigenic nature of this trait. As with any genetic analysis, it is theoretically possible that a closely linked gene or regulatory sequence[23], and not the identified gene itself, underlies the trait in question. The role of C5 itself in allergen-induced AHR is supported by *in vitro* functional data on the effects of C5 cleavage fragment signaling on immunoregulatory cytokine production, however.

It has become clear in recent years that the complement system plays an important immunoregulatory role at the interface of innate and acquired immunity. For example, proteolytic fragments of C3 have been shown to influence the class of immune response to a given immunogen, augmenting humoral immune responses through effects on B cells, and suppressing cellular immune responses through inhibition of IL-12 production by antigen presenting cells[24,25]. Because experimental allergic asthma is clearly a cytokine-regulated process, this suggested a mechanistic hypothesis for the role of C5-deficiency in susceptibility to allergen-induced AHR. Proteolytic cleavage of C5 yields two fragments, both of which can stimulate cytokine production. As part of a hemolytically inactive membrane attack complex, C5b causes signaling in neutrophils and endothelia, inducing chemokine production by the latter[26-28]. C5a has pleiotropic effects on inflammation, being chemotactic for all myeloid lineages, inducing degranulation and the production of variety of proinflammatory mediators by granulocytes and increasing vascular permeability[22]. C5a also stimulates monocyte and macrophage production of the proinflammatory cytokines TNF-α, IL-1 and IL-6[29-31]. We therefore examined the effects of C5a on monocytic production of IL-12. The data presented here demonstrate that blockade of C5aR signaling ablates IL-12 production by primary human monocytes. Such blockade also suppresses TNF-α production and reverses IFN-γ–mediated suppression of IL-10 secretion, although it does not affect bacterially driven IL-10 production.

C5a has itself been shown to lead to IL-12 inhibition under certain conditions[20,21], a finding that we were able to replicate. Our data demonstrate that such C5a-mediated IL-12 suppression is mechanistically distinct from the IL-12 suppression that results from the ablation of C5aR signaling, however. These data, together with previously reported data showing a lack of agonist effects of the C5aR antagonist[18], strongly suggest that the C5aR antagonist is not acting as an agonist at the receptor. Modulation of IL-12 by both the C5aR antagonist and C5a suggests a model in which some C5aR signaling is needed to render monocytes and macrophages competent for IL-12 production, whereas further exposure to C5a, especially in the presence of IFN-γ generated during an ongoing inflammatory process, leads to inhibition of the production of this potentially toxic cytokine.

Defective IL-12 production by C5a-deprived monocytes and macrophages provides a plausible mechanism for the regulation of susceptibility to asthma by C5: IL-12 drives type 1 $CD4^+$ T cell responses, preventing or reversing experimental allergic asthma. Furthermore, ablation of C5-mediated signaling releases monocyte and macrophages from IFN-γ–mediated inhibition of IL-10 production. Whereas IL-10 from antigen presenting cells can downmodulate both type 1– and type 2–polarized responses, feedback inhibition of IL-10 production by the type 1 cytokine IFN-γ itself promotes ongoing type 1 responses. As a consequence of these effects on cytokine production by antigen presenting cells, the absence of C5 is permissive for type 2 responses, including allergic asthma. Its presence promotes type 1 responses.

As predicted by studies with human monocytic cells, macrophages from C5-deficient A/J mice produce significantly less IL-12 than macrophages from C5-sufficient C3H/HeJ mice. The effect of *C5* genotype on maximal stimulation of IL-12 production by macrophages from these strains is not all-or-nothing. However, the differences in IL-12 productive capacity seen are biologically significant in the context of the *in vivo* development of an immunological response: IL-12 administration to A/J mice renders them resistant to the induction of asthma; neutralization of IL-12 in C3H/HeJ mice renders them susceptible[9]. Similar, biologically relevant, differences in IL-12 production by C5-deficient (A/J) and C5-sufficient (C57BL/6) strains have been found in a model of malaria infection[32]. Although a direct association between C5 deficiency and AHR in our model remains to be fully established, preliminary data from A/J mice in which the wild-type *C5* gene has been restored suggest that the T_H2-associated eosinophilic inflammatory response is attenuated in the presence of a functional *C5* gene.

These results provide insight into other models of genetic deletion or antibody-mediated inhibition of C5, C5a, or the C5aR. Such models have suggested a central role for C5 in the pathogenesis of collagen-

induced arthritis, DTH responses, and endotoxic shock, as well as in resistance to *Listeria* and to blood-stage malaria infection[32–36]. Although C5a has pleiotropic effects, including important effects on leukocyte trafficking, all of these models are dependent on or exacerbated by IL-12, and downmodulated by IL-10[37]. Likewise, *in vivo* deficiencies of C5 and the C5aR are associated with blunted production of TNF-α[38]. Given the important role of complement at the interface of innate and adaptive immunity, it is likely that complement-associated genes will provide high quality candidates for susceptibility to other immune-mediated diseases.

Our identification of *C5* as a susceptibility gene for experimental allergen-induced AHR may have relevance to human asthma. Two genome-wide screens for asthma susceptibility loci have found linkage to the *C5* chromosomal region (9q34)[5,6], and the human *C5aR* gene is located at 19q13.3, a chromosomal region with susceptibility loci for asthma in several different cohorts[4,5]. More generally, the likely role of C5-deficiency in suppressing the production of IL-12 in experimental asthma is echoed, pathophysiologically, by the finding that patients with allergic asthma have diminished production of IL-12 both in the lung and systemically[39,40]. Many of the candidate asthma susceptibility regions defined in human genetic studies contain genes whose products alter the balance of type 1 and type 2 cytokine expression. People with homozygous defects in the *C5* gene, like others with deficiencies in late complement components, suffer a high incidence of disseminated neisserial infection. They are protected, however, from the fulminant shock that often accompanies such disease in the face of complement-sufficiency, a phenomenon that may be due to the effects of a lack of C5a on TNF-α and IL-12 production[36]. Whether C5 null relatives are prone to develop allergic asthma does not appear to have been addressed.

The work reported here shows the utility of combined genetic and genomic approaches to the analysis of complex traits in experimental rodent models. Although genetic susceptibility loci for human diseases may not be direct homologs of loci identified in this fashion, genes encoding other proteins in the implicated biological pathways are likely candidates. In any case, rapid identification of susceptibility loci in experimental animal models is likely to provide substantial insight into pathogenetic mechanisms in human diseases. Even in the absence of full genomic information, the power of the approach to susceptibility gene discovery outlined here is evident. With full sequencing of the human and murine genomes currently in sight, these methods should find broad usage in the search for genetic susceptibility loci underlying complex human diseases.

Methods

Induction and characterization of the allergic phenotype. Four-week-old male mice (A/J, C3H/HeJ, AKR/J, BALB/c, C57BL6/J; n=4–14 mice per experimental group) and backcross mice (n=172) were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed under laminar flow hoods in an environmentally controlled specific pathogen-free animal facility for the duration of experiments. All mice were immunized by an intraperitoneal injection of 10 μg OVA (Sigma) in 0.2 ml PBS buffer. Mice were anaesthetized 14 days after immunization and challenged intratracheally with 50 μl of a 1.5% solution of OVA. Characterization of the allergic phenotype was performed 72 h after antigen challenge. Mice were anesthetized, intubated, ventilated at a rate of 120 breaths per min with a constant tidal volume of air (0.2 ml), and paralyzed. After establishment of a stable airway pressure, 50 μg per kg weight of acetylcholine was injected intravenously and dynamic airway pressure (APTI in cm-H_20 × s) was followed for 5 min.

Gene array assays. Gene chip arrays were hybridized with labeled cRNA obtained from whole lungs. Whole lungs, as opposed to cell type–specific subsets thereof, were employed to avoid bias: the relative importance to asthma pathogenesis of the multiple cell types that reside or traffic through the lung is unknown. Hybridizations were done with cRNA obtained from individual BC_1 mice; whereas lungs from A/J, C3H/HeJ and F_1 mice were pooled from four mice. Isolation of mRNA (2 × poly(A)$^+$), cRNA synthesis and hybridization of gene chips were performed as described in the *Affymetrix Expression Analysis Technical Manual*. The image was obtained with a 4x Image scan. Differential expression, as analyzed by GeneChip 3. 01 software from Affymetrix, Santa Clara, CA, was assessed by random pairwise comparisons of BC_1-high and BC_1-low responder mice, and by pairwise comparison of A/J and C3H/HeJ mice.

SNP assays. Genomic DNA was prepared from 176 phenotypically extreme BC_1 mice. The APTI values for BC_1-high responder mice were within the 90th percentile of APTI values from all BC_1 mice. The APTI values of BC_1-low responder mice were in the tenth percentile of all measured BC_1 mice. DNA from inbred murine strains was purchased from The Jackson Laboratory. Genotyping assays were performed by kinetic thermal cycling[41]. Allele-specific forward primers: *C5*-C3H/HeJ allele: AAGACATATTCTT TAATTTCAAAGTAT; *C5*-A/J allele: ACATATTCTTTAATTTCAAAGTGC; and a common reverse oligonucleotide primer: ACTATAAGAAGGATTTTACAACAACTGGAA; were used to amplify the strain specific *C5* alleles. Allele-specific forward primers: *Tlr4*-C3H/HeJ allele: CCTTCACTACAGAGACTTTATTCC; *Tlr4*-A/J allele: CCTTCAC TACAGAGACTTTATTCA; and a common reverse oligonucleotide primer: CCTGGAT GATGTTGGCAGCAA; were used to amplify the strain specific *Tlr4* alleles.

***In vitro* assays of human monocyte function.** Human monocytes isolated by countercurrent elutriation from normal volunteers[42] were cultured adherently in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD) at a density of 2×10^6 cells/ml (0.2 ml per data point) in 96-well plates (Costar, Cambridge, MA), in the presence or absence of varying concentrations of immunoaffinity-purified, bacterially expressed recombinant C5aR antagonist, or a control preparation similarly purified from bacteria not expressing C5aR antagonist. Isolation and culture of monocytes were performed under lipopolysaccharide (LPS)-free conditions. After 2–3 h in culture, monocytes were stimulated with IFN-γ 300U/ml (Pharmingen) followed 2 h later by *Staphalococcus aureus* Cowan strain I (SAC) 0.0075% (Calbiochem, La Jolla, CA), or with SAC alone. The production of p70 (as opposed to p40) by elutriated human monocytes is dependent upon preincubation with IFN-γ[43]. 24 h after SAC stimulation, cell-free culture supernatants were collected for measurement of cytokines by ELISA (IL-12 p40, IL-10, and TNF-α assays were from Pharmingen; IL-12 p70, from R&D Systems, Minneapolis).

All cell culture reagents were LPS-free to the limits of detection of the *Limulus* amebocyte lysate assay (3–6 pg/ml) (BioWhittaker, Walkersville, MD). Bactericidal/permeability increasing-protein (BPI) was employed in studies using complement reagents with measurable LPS contamination. At 5 μg/ml, the recombinant NH_2-terminal modified fragment of BPI (rBPI21; XOMA (US) LLC) has an LPS-neutralizing capacity of >10 ng/ml[44]. At experimental dilutions, all such reagents contained <100 pg/ml of LPS.

Acknowledgements

Supported in part by NIH grants AI40507, DE12167 (C.L.K.); ES09606, HL58527 (M.W.-K.); and RR00097 (S.L.E.), BMBF grant 01VM9305 (J.K.); a Michigan State University All-University Research Initiation Grant (S.L.E.); and EPA grant R826724 (M.W.-K.).The authors thank M. Shin for the gift of K-76.

Received 5 July 2000; accepted 3 August 2000.

1. Evans, R. D. *et al.* National trends in the morbidity and mortality of asthma in the US. Prevalence, hospitalization and death from asthma over two decades: 1965–1984. *Chest* **91**, 65S–74S (1987).
2. Wills-Karp, M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* **17**, 255–281 (1999).
3. Daniels, S. E. *et al.* A genome-wide search for quantitative trait loci underlying asthma. *Nature* **383**, 247–250 (1996).
4. A genome-wide search for asthma susceptibility loci in ethnically diverse populations. The Collaborative Study on the Genetics of Asthma (CSGA). *Nature Genet.* **15**, 389–392 (1997).
5. Ober, C. *et al.* Genome-wide search for asthma susceptibility loci in a founder population. The Collaborative Study on the Genetics of Asthma. *Hum. Mol. Genet.* **7**, 1393–1398 (1998).
6. Wjst, M. *et al.* A genome-wide search for linkage to asthma. German Asthma Genetics Group. *Genomics* **58**, 1–8 (1999).
7. Wills-Karp, M. *et al.* Interleukin-13: central mediator of allergic asthma. *Science* **282**, 2258–2261 (1998).
8. Gavett, S.H. *et al.* Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J. Exp. Med.* **182**, 1527–1536 (1995).
9. Keane-Myers, A., Wysocka, M., Trinchieri, G. & Wills-Karp, M. Resistance to antigen-induced airway hyperresponsiveness requires endogenous production of IL-12. *J. Immunol.* **161**, 919–926 (1998).
10. Ewart, S. E. *et al.* Identification of quantitative trait loci controlling allergen-induced airway hyperresponsiveness in inbred mice. *Am. J. Resp. Cell. Molec. Biol.*
11. Wetsel, R.A., Fleischer, D.T. & Haviland, D. L. Deficiency of the murine fifth complement component (C5). A 2-base pair gene deletion in a 5'-exon. *J. Biol. Chem.* **265**, 2435–2440 (1990).
12. Poltorak, A. *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science* **282**, 2085–2088 (1998).
13. Vogel, S. N. *et al.* Cutting edge: functional characterization of the effect of the C3H/HeJ defect in mice that lack an *lpsn* gene: *in vivo* evidence for a dominant negative mutation. *J. Immunol.* **162**, 5666–5670 (1999).
14. Johnson, E. & Hetland, G. Mononuclear phagocytes have the potential to synthesize the complete functional complement system. *Scand. J. Immunol.* **27**, 489–493 (1988).
15. Hindmarsh, E. J. & Marks, R. M. Complement activation occurs on subendothelial extracellular matrix *in vitro* and is initiated by retraction or removal of overlying endothelial cells. *J. Immunol.* **160**, 6128–6136 (1998).
16. Wozencraft, A. O., Sayers, G. & Blackwell, J. M. Macrophage type 3 complement receptors mediate serum-independent binding of *Leishmania donovani*. Detection of macrophage-derived complement on the parasite surface by immunoelectron microscopy. *J. Exp. Med.* **164**, 1332–1337 (1986).
17. Bergh, K. & Iversen, O. J. Production of monoclonal antibodies against the human anaphylatoxin

C5a des Arg and their application in the neoepitope-specific sandwich-ELISA for the quantification of C5a des Arg in plasma. *J. Immunol. Methods* **152**, 79–87 (1992).
18. Heller, T. *et al.* Selection of a C5a receptor antagonist from phage libraries attenuating the inflammatory response in immune complex disease and ischemia/reperfusion injury. *J. Immunol.* **163**, 985–994 (1999).
19. Hong, K., Kinoshita, T., Miyazaki, W., Inawa, T. & Inoue, K. An anticomplementary agent, K-76 monocarboxylic acid: its site and mechanism of inhibition of the complement activation cascade. *J. Immunol.* **122**, 2418–2423 (1979).
20. Wittmann, M. *et al.* C5a suppresses the production of IL-12 by IFN-γ -primed and lipopolysaccharide-challenged human monocytes. *J. Immunol.* **162**, 6763–6769 (1999).
21. Braun, M. C., Lahey, E. & Kelsall, B. L. Selective suppression of IL-12 production by chemoattractants. *J. Immunol.* **164**, 3009–3017 (2000).
22. Gerard, C. & Gerard, N. P. C5A anaphylatoxin and its seven transmembrane-segment receptor. *Annu. Rev. Immunol.* **12**, 775–808 (1994).
23. Loots, G. G. Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. *Science* **288**,136–140 (2000).
24. Fearon, D. T. The complement system and adaptive immunity. *Semin. Immunol.* **10**, 355–361 (1998).
25. Mosser, D. M. & Karp, C. L. Receptor mediated subversion of macrophage cytokine production by intracellular pathogens. *Curr. Opin. Immunol.* **11**, 406–411 (1999).
26. Wang, C. *et al.* Hemolytically inactive C5b67 complex: an agonist of polymorphonuclear leukocytes. *Blood* **85**, 2570–2578 (1995).
27. Wang, C., Gerard, N.P. & Nicholson-Weller, A. Signaling by hemolytically inactive C5b67, an agonist of polymorphonuclear leukocytes. *J. Immunol.* **156**, 786–792 (1996).
28. Kilgore, K. S. *et al.* Subiytic concentrations of the membrane attack complex of complement induce endothelial interleukin-8 and monocyte chemoattractant protein-1 through nuclear factor-κ B activation. *Am. J. Pathol.* **150**, 2019–2031 (1997).
29. Morgan, E. L. *et al.* Identification and characterization of the effector region within human C5a responsible for stimulation of IL-6 synthesis. *J. Immunol.* **148**, 3937–3942 (1992).
30. Schindler, R., Gelfand, J. A. & Dinarello, C. A. Recombinant C5a stimulates transcription rather than translation of interleukin-1 (IL-1) and tumor necrosis factor: translational signal provided by lipopolysaccharide or IL-1 itself. *Blood* **76**, 1631–1638 (1990).
31. Cavaillon, J.M., Fitting, C., Haeffner-Cavaillon, N. Recombinant C5a enhances interleukin 1 and tumor necrosis factor release by lipopolysaccharide-stimulated monocytes and macrophages. *Eur. J. Immunol.* **20**, 253–257 (1990).
32. Sam, H. & Stevenson, M. M. Early IL-12 p70, but not p40, production by splenic macrophages correlates with host resistance to blood-stage *Plasmodium chabaudi AS* malaria. *Clin. Exp. Immunol.* **117**, 343–349 (1999).
33. Wang, Y., Rollins, S. A., Madri, J. A. & Matis, L. A. Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease. *Proc. Natl Acad. Sci. USA* **92**, 8955–8959 (1995).
34. Tsuji, R. F. *et al.* Required early complement activation in contact sensitivity with generation of local C5-dependent chemotactic activity and late T cell interferon γ: a possible initiating role of B cells. *J. Exp. Med.* **186**, 1015–1026 (1997).
35. Gervais, F., Stevenson, M. & Skamene, E. Genetic control of resistance to *Listeria* monocytogenes: regulation of leukocyte inflammatory responses by the *Hc* locus. *J. Immunol.* **132**, 2078–2083 (1984).
36. Barton, P. A. & Warren, J. S. Complement component C5 modulates the systemic tumor necrosis factor response in murine endotoxic shock. *Infect. Immun.* **61**, 1474–1481 (1993).
37. Trinchieri, G. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv. Immunol.* **70**, 83–243 (1998).
38. Hopken, U. E., Lu, B., Gerard, N. P. & Gerard, C. Impaired inflammatory responses in the reverse arthus reaction through genetic deletion of the C5a receptor. *J. Exp. Med.* **186**, 749–756 (1997).
39. van der Pouw Kraan, T. C. *et al.* Reduced production of IL-12 and IL-12-dependent IFN-γ release in patients with allergic asthma. *J. Immunol.* **158**, 5560–5565 (1997).
40. Naseer, T. *et al.* Expression of IL-12 and IL-13 mRNA in asthma and their modulation in response to steroid therapy. *Am. J. Respir. Crit. Care Med.* **155**, 845–851 (1997).
41. Germer, S., Holland, M. J. & Higuchi, R. High-throughput SNP allele-frequency determination in pooled DNA samples by kinetic PCR. *Genome Res* **10**, 258–266 (2000).
42. Karp, C. L. *et al.* Mechanism of suppression of cell-mediated immunity by measles virus. *Science* **273**, 228–231 (1996).